# Chapter 7

# The Hill Reaction: In Vitro and In Vivo Studies

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## Contents



## Introduction

These exercises attempt to bridge the relationship between light absorption and the utilization of that energy. The first exercise shows that radiant energy is required by chloroplasts to reduce artificial electron donors and that photosynthetic herbicides block this electron flow. The second exercise shows that chlorophyll in solution, when it does not participate in chemical reactions, fluoresces red, either when irradiated with white light or UV light. The third exercise ties these two observations together. When intact cells are irradiated wit UV light, there is little chlorophyll fluorescence, because most of the absorbed energy is consumed in the photochemistry of the light reactions. When that photochemistry is inhibited by the herbicides, the absorbed energy is readmitted as red fluorescence. For further reading, see Balint and Funkhouser (1993), Funkhouser and Sherman (1987), and Salisbury and Ross (1992).

## Student Outline

## Introduction

"*Sunshine splits apart the carbon dioxide that a plant has absorbed from the air, the plant throws out at that time the oxygen alone and keeps the carbon to itself as nourishment.*" — Jan Ingenhousz (1779)

So firmly was this belief entrenched that in several European languages the term "carbon dioxide assimilation" also meant what is now called photosynthesis. This seemingly logical structure was undermined in the 1880s by Theodor Engelmann at the University of Utrecht. He found that purple bacteria which metabolize sulfur compounds perform a type of photosynthesis apparently without giving off oxygen. As so often happens when an experimental result contradicts the prevailing fundamental notions, the implications of this discovery were simply ignored.

It took 40 years and many more experiments to establish the significance of chemosynthetic and photosynthetic bacteria. C. B. van Niel, in the early 1930s conclusively demonstrated that bacteria can carry on photosynthesis without evolving oxygen. This suggested to van Niel that the $O_2$ released by plants is derived from water, not $CO_2$.

Van Niel's idea was supported in the late 1930s by the work of Robin Hill and R. Scarisbrick. They showed that an inorganic substance, ferricyanide, was reduced to ferrocyanide when extracellular preparations made from plant cells were illuminated. At the same time water was oxidized to oxygen. Their results meant that isolated chloroplasts and chloroplast fragments could release $O_2$ in light if they were given a suitable acceptor for the electrons released from the light-driven splitting (*photolysis*) of water. In vivo, these electrons ultimately reduce $NADP^+$ to

NADPH. The photolysis of water in the absence of $CO_2$ fixation became known as the **Hill Reaction**. It is associated with Photosystem II (see Figure 7.1). In order to observe Hill activity, reaction vessels must contain functional chloroplast membranes, water, and an electron acceptor. The rate of this reaction is then dependent on light intensity.

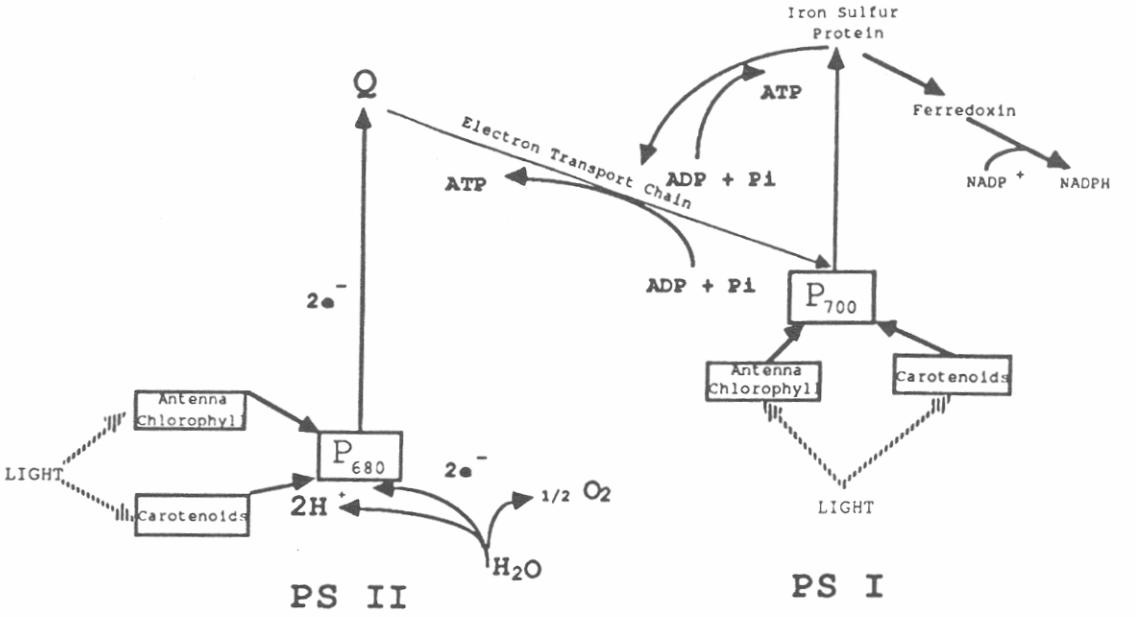


**Figure 7.1.** Z-scheme for photosynthesis.

### Exercise 1: Hill Activity in Chloroplasts

**Purpose:** To observe the effect of light intensity on Hill Reaction activity of isolated chloroplast particles and to observe the effect of herbicides on this activity.

In this exercise we will use dichlorophenol indophenol (DCPIP) as the electron acceptor of Photosystem II activity. Oxidized DCPIP is blue while reduced DCPIP is colorless. The progress of the reduction can be monitored by the disappearance of the blue color in the reaction vessels.

$$\underset{\text{(blue)}}{\text{DCPIP}} + H_2O \xrightarrow[\text{light and chloroplasts}]{} \underset{\text{(colorless)}}{\text{DCPIP-}H_2} + O_2$$

(*Remember*: Chloroplasts are green so the tubes will never become clear!)

The rate of oxygen release parallels the rate of DCPIP reduction which will be monitored colorimetrically at 600 nm. This exercise will show the kinetics of the reaction, its dependence on light, and effect of the herbicide Diuron.

#### Materials

(The first six items are used for the chloroplast suspension.)

Spinach leaves (25 g)
Sucrose, 0.5 M, cold (120 ml)
Chilled blender
Cheesecloth
Centrifuge and centrifuge tubes
Phosphate buffer (pH 6.5), 0.10 M, cold (240 ml)
Colorimeter
Cuvettes and rack (11)
Parafilm squares, 1" (10)
DCPIP, 0.2 mM (10 ml)
Phosphate buffer (pH 6.5), 0.1 M (10 ml)
$H_20$ (20 ml)
Diuron, 0.10 mM (2 ml)
Diuron, 0.01 mM (2 ml)
Pipets, 1.0 ml (3)
Pipets, 2.0 ml (3)
Foil squares, 3" (2)
Light source
Heat sinks (3)
Test tube racks (6)

*Caution*: All reaction vessels which contain chloroplasts should be kept in the dark except when measurements are taken and during light exposure. This can be accomplished by placing the test tubes in drawers. *Also, do not pipet by mouth!*

## Procedures

*I – Preparation of Chloroplast Suspension*

The laboratory instructor has prepared a suspension of chloroplasts for you. It was prepared as follows: spinach leaves were de-ribbed and washed and then homogenized in a cold sucrose solution in a chilled blender (2 × 10 second bursts). The homogenate was filtered through four two-layers of cheesecloth and centrifuged for 10 minutes at 8000 × g. The resulting pellet was resuspended in a cold phosphate buffer. This preparation should produce a rate of 0.2 per minute for reaction cuvette #4. *Keep the chloroplast suspension in the dark and on ice prior to use.*

*II – Kinetics of DCPIP Reduction*

There are several ways in which the rate of reduction of DCPIP can be determined. One is to continuously monitor the disappearance of the blue pigment. Technically this is very difficult if, at the same time of measurement, the reaction vessel needs to be exposed to light. *If it can be shown that the rate of reduction is linear with time, then all that is necessary is two points to draw the straight line.* Two convenient points are the beginning and end of the incubation. This part of the exercise will show that, initially, the rate of reduction is linear with time.

1. Add 1 ml DCPIP, 1 ml buffer, and 2 ml water to cuvette #1.
2. Add 1 ml of chloroplast suspension, invert several times and read absorbance. This reading should range between 0.75 and 0.90. (*Note:* Absorbance will be read at 600 nm throughout the entire exercise.)
3. Place the cuvette at position #4 at the light source (see Figure 7.2). Read and record the absorbance at 2 minute intervals for 14 minutes. (Wrap the cuvette in foil while transporting it

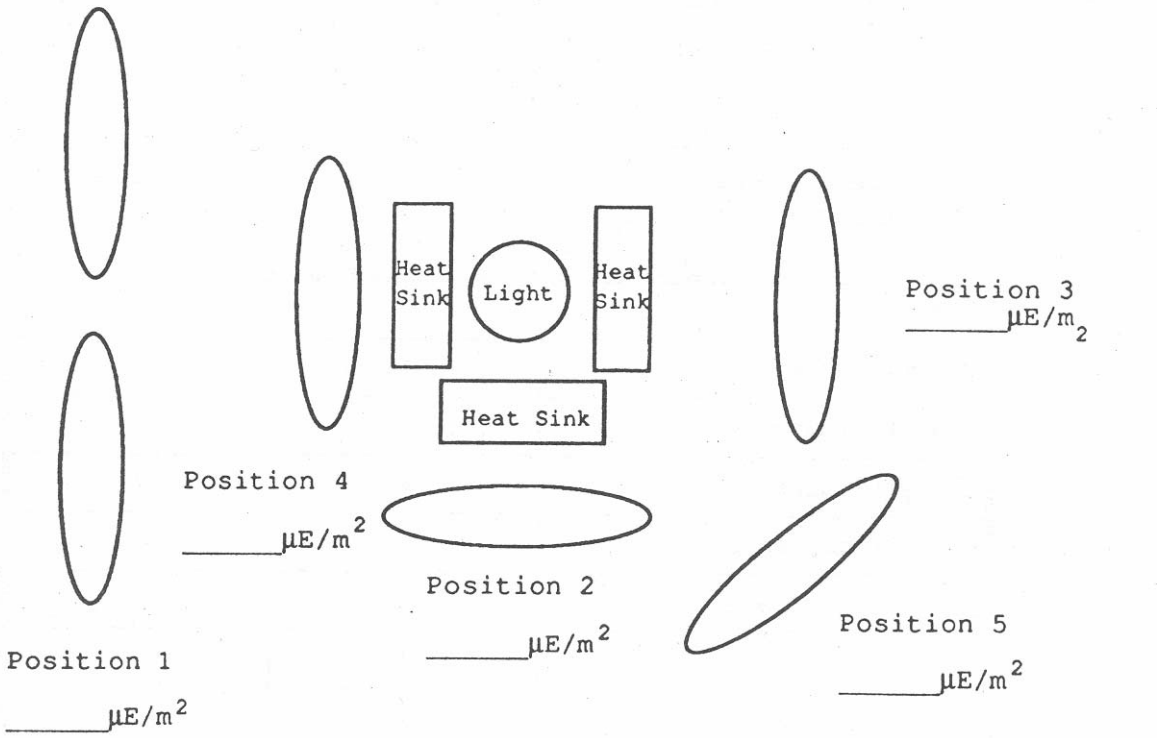


**Figure 7.2.** Experimental layout.

*III – Effect of Light Intensity on Hill Activity*

By placing reaction vessels varying distances from a light source, the light intensity reaching each vessel is different. Test tube racks have been positioned around a light source (see Figure 7.2). The light intensity at each position was measured by the instructor. This information should be recorded in Figure 7.2. If the rate of reduction is light dependent, rates in vessels far from the light source should be slower than those close to the light source. This exercise will test this hypothesis.

1. Omitting the chloroplasts, prepare six cuvettes (#2–7) as in step 1 of Part II. Invert cuvette #2 several times and record its absorbance.
2. Add 1 ml of chloroplasts to cuvette #3. Immediately after the addition of chloroplasts, invert the cuvette several times and record the absorbance. Repeat for cuvettes #4 through #7.
3. After each absorbance measurement, place each cuvette in the dark until the absorbance of all cuvettes are determined.
4. Place the six cuvettes in the proper positions around the light source. (Cuvette #3 should be wrapped in foil and may be kept in the drawer.)
5. After 10 minutes record the final absorbance. Results can be recorded on Table 7.1.

*Note*: Cuvettes #2 and #3 are controls.

**Table 7.1.** Absorbance (*A*) values.

| Cuvette number and treatment | Light position | Time | $A_{600}$ initial | $A_{600}$ final | $A_{600}$ Δ |
|---|---|---|---|---|---|
| 2. Control | 1 | | | | |
| 3. Dark control | — | | | | |
| 4. Light treatment 1 | 2 | | | | |
| 5. Light treatment 2 | 3 | | | | |
| 6. Light treatment 3 | 4 | | | | |
| 7. Light treatment 4 | 5 | | | | |
| 8. Control | 2 | | | | |
| 9. 0.01 mM DCMU | 2 | | | | |
| 10. 0.10 mM DCMU | 2 | | | | |

**Note*: Cuvette #1 was used to show linearity of reaction.

*IV – Effect of Herbicides on Hill Reaction Activity*

Several herbicides act by inhibiting photosynthesis. One way that photosynthesis can be inhibited is by blocking electron flow between the two photosystems (Figure 7.1). By varying the concentration of the herbicide Diuron (DCMU), this inhibitory effect can be readily demonstrated.

1. Dilute the stock solutions of DCMU to the concentrations indicated in Table 7.1. (This can be easily accomplished with a serial dilution.)
2. Omitting chloroplasts, prepare cuvettes #8 to #10 in the following manner:

   #8: 1 ml of buffer, 1 ml of DCPIP, and 2 ml of water
   #9: 1 ml of buffer, 1 ml of DCPIP, and 2 ml of 0.01 mM DCMU
   #10: 1 ml of buffer, 1 ml of DCPIP, and 2 ml of 0.10 mM DCMU

3. Add 1 ml of chloroplasts to one cuvette, invert several times, and record the absorbance (as in step 2 of Part III). Repeat with remaining cuvettes.
4. After each absorbance measurement, place each cuvette in the dark until the absorbance of all tubes has been determined.
5. Place all cuvettes together under the light bank at position #2 (see Figure 7.2).
6. When cuvette #8 (control) has changed color from blue to green (1 to 10 minutes), move all tubes to the dark (drawer).
7. Record the time exposed to light, and the final absorbance of each cuvette on Table 7.1.
8. Calculate the rate of DCPIP reduction as the change in absorbance at 600 nm per 10 minute.

**Report**

1. Cuvette #1: Plot Absorbance as a function of time. (There should be a linear relationship.) Discuss the progress of DCPIP reduction in light.
2. Cuvettes #2–7: Plot rate as a function of light intensity. Discuss the relationship between light intensity and the rate of the Hill Reaction.
3. Cuvettes #8–10: At each herbicide concentration calculate the percent inhibition of DCPIP reduction. Control rate (tube #8) is 100% activity (0% inhibition). Any treatment with no change in absorbance is 0% activity (100% inhibition). Plot per cent inhibition as a function of the logarithm of inhibitor concentration. Discuss the relationship between inhibitor concentration and the per cent inhibition.

**Questions**

1. For how long was the rate of reduction linear in cuvette #1?
2. Does your data indicate that the highest light intensity saturated the rate of the Hill Reaction?
3. Where on the "Z" scheme, does DCPIP and the inhibitor interrupt the electron flow?
4. What is the terminal electron acceptor in the light reactions in intact chloroplasts?

## Exercise 2: Chlorophyll Fluorescence

**Purpose:** To observe chlorophyll fluorescence.

Chlorophyll, as the primary light-absorbing pigment in chloroplasts, traps radiant energy. When chlorophyll absorbs this energy, the pigment is excited to higher energy levels which drive the photochemistry. The energy of excited chlorophyll is utilized to oxidize water to molecular oxygen with the conominant formation of reducing equivalents. If this photochemistry is inhibited, the energy of the excited chlorophyll molecules can be released in the form of re-emitted light or fluorescence of characteristic wavelength.

### Materials

Spinach leaves, fresh (10 g)
Acetone (100 ml)
Mortar and pestle
Funnel and filter paper
Beaker, 250 ml
Bottle with cap, 100 ml
UV light source
White light source (slide projector with slit mask over lens)
Eye protection

### Procedures

1. Weigh 10 g of de-veined spinach.
2. Grind in mortar and pestle with 50 ml of acetone; filter into beaker.
3. Re-extract residue with another 50 ml of acetone; filter, and pool with first extraction.
4. Pour into bottle and cap.
5. In a darkened room, view bottle with white light from several angles.
6. In a darkened room, view bottle with UV light. *(Wear eye protection.)*

### Report

1. When viewed with white light, what color was transmitted? Why?
2. When viewed with white light, what color was observed 90° to the incident light? Why?
3. When viewed with UV light, what color was observed? Why?

## Exercise 3: Hill Activity in Cells

**Purpose:** To demonstrate the effect of triazine herbicides on photosynthesis within whole cell.

Many herbicides act as inhibitors of photosynthesis. The triazine herbicides attach to specific proteins in thylakoids and thereby block electron transport between the photosystems. In the previous exercise, this effect was demonstrated as an inhibition of Hill activity when isolated chloroplasts were treated with Diuron (a triazine herbicide). This exercise demonstrates the same effect, using *Chlorella* instead of isolated chloroplasts.

Under normal conditions, the energy of photosensitized chlorophyll molecules is used to support the chemistry of the light reactions of photosynthesis. When electron transport between the two photosystems is inhibited, energy of activated chlorophyll is lost in the form of fluorescence and heat. Therefore, plants which have been treated with photosynthetic inhibitors fluoresce more than untreated controls.

### Materials

*Chlorella* culture (50 ml)
Erlenmeyer flasks, 25 ml (2)
Water (1 ml)
Diuron, 1 mM (1 ml)
Pipet, 20.0 ml (1)
Pipets, 1.0 ml (2)
Parafilm squares, 1" (2)
Light source
UV light source with a red filter
Eye protection

### Procedures

1. Remove about 50 ml of *Chlorella* culture from the stock culture.
2. Place 20 ml of culture in each of two 25 ml flasks.
3. To one flask add 1 ml of water, and to the other, add 1 ml of Diuron. *Do not pipet by mouth!*
4. Cover both flasks with parafilm, mix well, and then place under the light bank. After 5 to 15 minutes, remove the two flasks from the light source and examine in a darkened room under UV light with a red filter. *(Wear eye protection.)*

### Report

1. Which flask showed the greater fluorescence?
2. Why was it necessary to use a darkened room in order to observe the fluorescence?
3. What would you expect to see if leaves were “painted” with a solution of the herbicide?
4. How would this information about herbicide-induced fluorescence be beneficial in a program for breeding herbicide resistance in crop plants?

## Notes for the Instructor

*Exercise 1:* Arrange test tube racks around a light source (usually a 100–150 watt bulb) in a dark room with a heat sink between the light source and the racks. Chromatography tanks work well. It is helpful to have the light source plugged into a rheostat. If rates are too fast, reduce light intensity.

The first part of the exercise is to show the linearity of the reaction. Intensity should be intermediate so that the rate is linear for at least 10 minutes, but not more than 20 minutes. Reaction rates should be linear with intensity under these conditions.

Atrizine, or another triazine herbicide, can be substituted for Diuron (3-(3,4-dichlorophenyl)-1,1-dimethyl urea). Diuron can be purchased from Sigma Chemical Company.

*Exercise 2:* Any commercial "black light" can be used as a light source. Pocket flashlights can be used as sources of white light for individual observations. The exercise can be expanded to larger containers for demonstration in a lecture hall (with the hall lights off).

*Exercise 3:* Just about any moderately dense solution of single-celled algae will work. Refer to Funkhouser and Sherman (1987) for details on culturing methods. Cells with short or long doubling times have been used successfully. Cultures of 1-liter in a 2-liter flask work well as a demonstration in a lecture hall when there is little light.

## Literature Cited

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